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Human serum albumin and its structural variants mediate cholesterol efflux from cultured endothelial cells

Ji-Sook Ha[a], Chung-Eun Ha[a], Jun-Tzu Chao[b,1], Charles E. Petersen[a,2], Andre Theriault[b], Nadhipuram V. Bhagavan[a,*]

[a] *Department of Biochemistry and Biophysics, John A. Burns School of Medicine, University of Hawaii at Manoa, 1960 East–West Road, Honolulu, HI 96822, USA*

[b] *Division of Medical Technology, John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, HI 96822, USA*

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Abstract

In the present study, we used the human EA.hy926 endothelial cell line as the model system to investigate the effect of human serum albumin (HSA) and its structural variants on cholesterol efflux. Initial studies showed that HSA promoted cholesterol efflux in a dose- and time-dependent manner, reaching a plateau at 10 mg/ml at 90 min. As a control, gelatin displayed no significant effect on efflux, while HSA was significantly more efficient than ovalbumin and bovine serum albumin (BSA) in promoting cholesterol efflux. Equal molar concentrations of HSA and apolipoprotein A-I (apoA-I) showed that apoA-I had considerably higher efficiency in efflux. However, the prevailing high plasma concentrations of HSA may compensate for its lower efflux rate compared to apoA-I. To characterize the mechanism of HSA-mediated cholesterol efflux, we studied the effects of cAMP and temperature on efflux using both EA.hy926 endothelial cells and murine RAW 264.7 macrophages. We found that HSA-mediated efflux occurred via a cAMP-independent and relatively temperature-insensitive pathway. We next examined the nature of HSA–cholesterol interaction by comparing the effects of various HSA mutants to wild-type HSA on cholesterol efflux. We found specific interactions between subdomains 2A and 3A and cholesterol, as indicated by the changes in the efflux rate of various HSA mutants. In conclusion, our study provides evidence for the role of HSA in cholesterol efflux, and shows that the substitution of specific amino acid residues in subdomains of 2A and 3A may be important structural determinants in its ability to bind to cholesterol and participate in cholesterol efflux.

Keywords: Cholesterol efflux; Human serum albumin; Ligand binding; Site-directed mutagenesis; Endothelial cell; RAW 264.7

1. Introduction

Human serum albumin (HSA) is a major protein component of plasma. It is mainly responsible for maintaining plasma oncotic pressure and binding of various endogenous and exogenous ligands including hormones, toxic metabolites, and various drugs among other hydrophobic ligands [1–3]. By binding to various ligands in circulation, HSA modulates hormonal activity, toxicity of various endogenous and exogenous substances, and drug availability. Various ligand binding studies, including X-ray crystallography, on HSA have shown that it has two major ligand binding sites located in the subdomains of 2A and 3A [4–6]. Thyroxine, bilirubin, warfarin, among many others, are examples of HSA ligands that bind to these sites. Another possible ligand may be cholesterol whose interaction with HSA has not been investigated.

Abbreviations: HSA, human serum albumin; wtrHSA, recombinant wild-type HSA; BSA, bovine serum albumin; CPT-cAMP, 8-(4-Chlorophenylthio) adenosine3′:5′-cyclic monophosphate; apoA-I, apolipoprotein A-I; ABCA1, ATP binding cassette A1; CHD, coronary heart disease; HDL, high-density lipoprotein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HUVEC, human vein endothelial cell; PBS, phosphate-buffered saline, 137 mM NaCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 2.7 mM KCl, pH adjusted to 7.4 with HCl; OVA, chicken ovalbumin

* Corresponding author. Tel.: +1-808-956-8130; fax: +1-808-956-9498.

E-mail address: bhagavan@hawaii.edu (N.V. Bhagavan).

[1] Present address: Department of Medical Physiology, the Texas A&M University System Health Science Center, 336 Reynolds Medical Building, College Station, TX 77843-1114, USA.

[2] Present address: Chemical Diversity Laboratories, Inc., 11558 Sorrento Valley Road, Suite #5, San Diego, CA 92121, USA.

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Previous studies have shown that HSA mediates cholesterol efflux from cultured human fibroblasts [7–9]. Also, an animal study in rats showed that serum albumin played a major role in cholesterol transport in circulation and estimated approximately 24% of the nonesterified cholesterol was bound to albumin [10]. These observations were pivotal in pursuing our study. HSA, like high-density lipoprotein (HDL), may participate in the reverse cholesterol transport from peripheral cells. HSA-mediated cholesterol efflux may have two important attributes, which may contribute to its superior activity over the well-studied apolipoprotein A-I (apoA-I)-mediated efflux. First, it has a higher capacity for transport due to relatively high concentrations of HSA (660 μM) in human plasma compared to apoA-I (55 μM), and secondly, its polymorphic forms may possess a higher affinity for cholesterol. In regard to the second attribute, HSA mutant proteins can be used as potential binding agents to remove excess cholesterol from circulation as similarly described for digitalis glycoside, bilirubin, and warfarin [11–13]. Site-directed mutagenesis studies of HSA have shown that various point mutations introduced into major ligand binding domains of HSA induces significant changes in its ligand binding capacity [13–16], with the possible result of affecting metabolism of certain ligands. The 2A and 3A subdomains of HSA have been shown to be two major sites in hydrophobic ligand binding, such as fatty acids, warfarin, bilirubin, and diazepam [17]. A number of studies have shown that steroids bind to HSA with moderate binding affinity and that fatty acids can displace up to 50% of steroids bound to HSA, indicating that steroids are likely to bind in the 2A or 3A subdomain [18]. Since cholesterol is a steroid, we hypothesized that all steroid molecules may share the same binding domain of HSA. Thus, we used a number of 2A and 3A subdomain mutants to study the effects of various amino acid substitutions on cholesterol efflux. Since several epidemiologic studies have shown an inverse relationship between serum HSA levels and the incidence of coronary heart disease (CHD) [19–25], one of the aims of our cholesterol efflux study is to investigate a possible mechanism for this inverse relationship. Since one of the principal cells involved in cholesterol efflux are endothelial cells, we undertook this study using primarily an immortalized human endothelial cell line, EA.hy926, as the model system. We also used murine RAW 264.7 macrophages to study the mechanisms involved in the efflux process by HSA.

2. Methods

2.1. Materials

Immortalized human vein endothelial cells (EA.hy926) were generously provided by Dr. Cora-Jean S. Edgell (University of North Carolina, Chaphill, NC) and murine RAW264.7 macrophages were obtained from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Hank's buffer saline solution, and all other tissue culture supplies were purchased from Invitrogen Life Technologies Inc. (Grand Island, NY). Bovine serum albumin (BSA, fatty acid free), HSA (fatty acid free), ovalbumin, gelatin, type I collagen, 8-(4-Chlorophenylthio) adenosine3′:5′-cyclic monophosphate (CPT-cAMP) and Cibacron coupled to Sepharose 6B were purchased from Sigma Chemical Co. (St. Louis, MO). Human apoA-I was purchased from Calbiochem (La Jolla, CA). 1-2n-[^{3}H]cholesterol (specific activity 40–60 Ci/mmol) was from Perkin Elmer Life Science Research Products (Norwalk, CT). Vent DNA polymerase was from New England Biolabs (Beverly, MA). Plasmid vector, pHiL-D2 and a yeast strain, *Pichia pastoris* were purchased from Invitrogen Corp. (San Diego, CA). Lipidex-1000 was from Packard Instruments (Meriden, CT). BCA protein assay kit for quantification was from Pierce Co. (Rockford, IL).

2.2. Synthesis and purification of recombinant HSA and its various mutants

For our studies, we synthesized recombinant wild-type HSA (wtrHSA) and the following single and double mutants that were chosen based upon earlier studies of steroids and digoxin binding to HSA mutant proteins [13]. The mutants studied were K195M, K199M, F211V, W214L, R218A, R218M, F242V, R257M, Y411A, R410A, R410M, R410Q/Y411W, R410M/Y411F, Y411F, Y411L, W214L/Y411W, and R410A/Y411A.

Specific mutations were introduced into the HSA-coding region in a plasmid vector containing the entire HSA coding region as described previously [14,16,26]. The experimental methods consist of the following steps.

2.2.1. Cloning of HSA coding region

With human liver cDNA as the template, the entire coding region of the HSA gene, including the native signal sequence, was amplified by polymerase chain reaction using Vent DNA polymerase. The resulting DNA fragment was inserted into the plasmid vector pHiL-D2 using standard cloning techniques. pHiL-D2 is a shuttle vector that can be manipulated by cloning in *Escherichia coli* and that can also be used to introduce genes into yeast species *P. pastoris* by homologous recombination. Specific mutations were introduced into the HSA coding region using site-directed mutagenesis as described previously [14,16,26].

2.2.2. Expression of recombinant HSA

Each pHiL-D2 expression plasmid contained a methanol-inducible promoter upstream of the HSA coding region. For each expression plasmid, a yeast clone that contained the expression cassette stably integrated into the yeast chromosomal DNA was isolated. The native HSA signal

sequence, which was left on the HSA coding region, directed high-level secretions of mature HSA into the growth medium.

2.2.3. Verification of DNA sequence of HSA clones

The total genomic DNA from each *P. pastoris* clone used to produce a particular HSA species was isolated using standard techniques. The genomic DNA isolated from each clone was used as template to amplify the entire HSA coding region by polymerase chain reaction. For each clone, the entire HSA coding region was sequenced using the dideoxy chain termination technique, and the translation product corresponding to this sequence matched a previously published cDNA of HSA at all amino acid positions except for the mutation introduced into a particular HSA mutant.

2.2.4. Purification of recombinant HSA

The secreted HSA was isolated from the growth medium as follows. The medium was brought to 50% saturation with ammonium sulfate at room temperature. The temperature was then lowered to 4 °C, and the pH was adjusted to 4.4, the isoelectric point of HSA. The precipitated protein was collected by centrifugation and resuspended in distilled water. Dialysis was carried out for 48 h at 4 °C against 100 volumes of distilled water, followed by 24 h against 100 volumes of phosphate-buffered saline (PBS, 150 mM NaCl, 40 mM phosphate, pH 7.4). The solution was loaded onto a column of Cibacron blue immobilized on Sepharose 6B. After washing the column with 10 bed volumes of PBS, the HSA was eluted with 3 M NaCl. The eluent was dialyzed into PBS and passed over a column of Lipidex-1000 to remove hydrophobic ligands possibly bound to the HSA [14]. The resulting protein migrated as a single band on SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Cholesterol efflux

2.3.1. Cell culture

EA.hy926 endothelial cells, seeded in 96 collagen-coated wells, were maintained in DMEM supplemented with 10% FBS, 0.1 mM sodium hypoxanthine, and 16 μM thymidine at 37 °C, 5% CO_2 and 95% air. This specific cell line was derived from human umbilical vein endothelial cell (HUVEC) by fusion with a relatively undifferentiated A549/8 cell line which has been characterized to possess normal human endothelial cell properties [27–30]. Murine RAW264.7 macrophages were also grown and maintained in DMEM supplemented with 10% FBS. Upon reaching confluency, cells were labeled with [^{3}H]cholesterol and assayed for cholesterol efflux.

2.3.2. [^{3}H]cholesterol efflux assay

The labeling media was first prepared by concentrating 1-2n-[^{3}H]cholesterol (40–60 Ci/mmol) under vacuum and resolubilizing in ethanol so that the final concentration of ethanol did not exceed 0.1% of the final media concentration. This [^{3}H]cholesterol–ethanol solution (20 μCi/ml) was dissolved into the culture medium supplemented with 5% FBS and added to the cells for 48 h to equilibrate cellular cholesterol pools. Efflux was carried out by washing the labeled cells twice with DMEM containing 2 mg/ml BSA and twice with DMEM alone. The washed radiolabeled cells were incubated at 37 °C with DMEM in the presence or absence of various cholesterol acceptors for various time intervals, using an orbital shaker. At the indicated time interval, 50 μl of incubation medium was taken and mixed with 100 μl of PBS containing 2 mg/ml BSA. The mixture was centrifuged at 10,000 rpm, 5 min and 100 μl of supernatant was used for radioactivity counting. At the end of incubation, cells were washed once with PBS containing 2 mg/ml BSA and twice with PBS alone followed by lysis with 0.2 ml of 0.1 N NaOH. Aliquots were taken for radioactivity counting by liquid scintillation counter (Packard Tri-Carb 1500 liquid Scintillation System) and cellular cholesterol efflux was expressed as cpm/mg cell protein, as well as percent of total cholesterol released into medium (percent efflux=(medium cholesterol/cell + medium cholesterol)100). Total protein concentration was determined according to the modified Biuret protein assay method.

2.4. Statistical analysis

All data were presented as mean ± S.E. Statistical differences were analyzed using analysis of variance (ANOVA) or paired *t*-test with the level of significance set at 0.05.

3. Results

3.1. HSA stimulates cholesterol efflux in a concentration- and time-dependent manner

Initial studies were performed to determine an optimal concentration of HSA and incubation time on cholesterol efflux. After 48 h of [^{3}H]cholesterol labeling, cells were replenished with media containing various concentrations of HSA. An aliquot of medium was taken at the 30-, 60-, and 90-min interval of the incubation, and the radioactivity was measured. As illustrated in Fig. 1, HSA promoted cholesterol efflux in a concentration- and time-dependent manner within an optimal concentration of 10 mg/ml at 90 min. At the 10 mg/ml concentration, efflux increased 4-fold at 30 min, 9-fold at 60 min, and 12-fold at 90 min compared to the DMEM control, respectively. At the 90-min incubation time, efflux increased 8-fold at 1 mg/ml HSA concentration, 10-fold at 5 mg/ml, 12-fold at 10 mg/ml, and 18-fold at 40 mg/ml. Efflux became less responsive at HSA concentration of 40 mg/ml or 588 μM, which approximated the physiological concentration of HSA at all time intervals. Thus, in

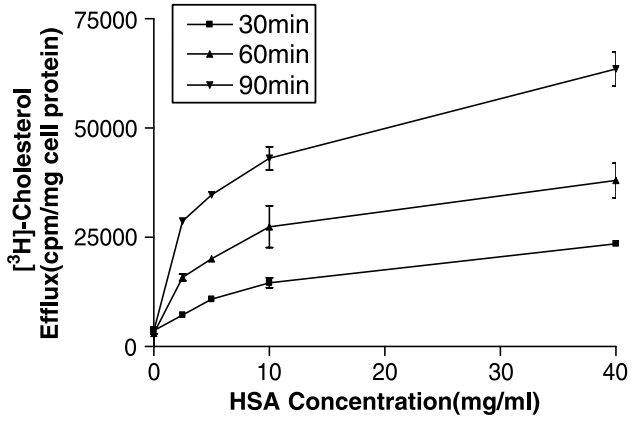


Fig. 1. Effect of various HSA concentrations and incubation time on cholesterol efflux. Endothelial cells were cultured and labeled with [^{3}H]cholesterol (20 μCi/ml) for 48 h. Cells were washed and incubated with DMEM containing various amounts of HSA (2.5, 5, 10, 40 mg/ml) for different incubation times (30, 60, and 90 min). Aliquots of the medium were taken at the designated time intervals to assess cholesterol efflux. Results are expressed as radioactivity (cpm) released into the media per milligram of cell protein. The values represent the mean ± S.E. from three independent experiments performed in triplicate.

all further experiments, 10 mg/ml HSA and 90-min incubation time were used.

3.2. HSA-mediated cholesterol efflux is specific

In order to determine whether the efflux observed from endothelial cells is the result of a specific interaction between HSA and cholesterol, we investigated the effects of BSA, chicken ovalbumin (OVA) and gelatin on cholesterol efflux. After 48 h of labeling, cells were replenished with media containing 10 mg/ml of HSA, BSA, ovalbumin, or gelatin. An aliquot of medium was taken at 90-min interval of the incubation, and the radioactivity was measured. As shown in Fig. 2, HSA significantly increased cholesterol efflux compared to untreated control (DMEM) at both time intervals. On the other hand, gelatin, a matrix protein, displayed little effect on cholesterol efflux, while BSA and ovalbumin showed a small degree of stimulation compared to the untreated DMEM control (gelatin: 1.3-fold; ovalbumin: 3.4-fold; BSA: 7.9-fold higher efflux vs. DMEM control at the 90-min incubation time). The differences in cholesterol efflux observed between BSA and HSA is presumably due to a 76% amino acid sequence homology and thus provides an explanation for the different cholesterol efflux efficiency between HSA and BSA [1]. However, ovalbumin does not have sequence homology with HSA but its high content of α-helixes may have contributed to the slight increased in cholesterol efflux. Taken together, the effect of HSA on cholesterol efflux appears to be a specific event.

The ability of apoA-I to promote cholesterol efflux from cultured cells is well documented [8,31–34]. To estimate the degree of HSA's contribution toward cholesterol efflux, we compared the efflux rate mediated by HSA to that of apoA-I (Fig. 3). As expected, reconstituted apoA-I exceeded cholesterol efflux efficiency compared to equal molar concentration (i.e. 1 μM) of wtrHSA (168 ± 2%, $P < 0.05$ vs. HSA). The data indicates that apoA-I is a major cholesterol acceptor and possibly a major contributor toward peripheral cholesterol efflux. However, as previously mentioned, we believe that HSA may be as important as apoA-I in cholesterol efflux because of its high plasma concentration. The approximate mean HSA concentration in human plasma is 600 μM in contrast to apoA-I which is 55 μM. To determine whether HSA and apoA-I-mediated efflux is additive to each other, we incubated cells in the media containing equimolar concentration of HSA and apoA-I as cholesterol acceptor. Interestingly, HSA- and apoA-I-mediated cholesterol efflux showed that two processes are additive, indicating that the two cholesterol efflux systems employ totally separate processes.

3.3. HSA-mediated cholesterol efflux occurs via a cAMP-independent and a relatively temperature-insensitive pathway

At least two independent peripheral cholesterol efflux pathways have been described [31]. One is the apoA-I-mediated process, which is regulated by lipid-poor HDL and is cAMP dependent. This pathway involves the transport of cholesterol via ABC (ATP-binding cassette transporter) A1. The other pathway is a nonspecific diffusion-mediated cholesterol efflux from cell surface. This pathway is regu-

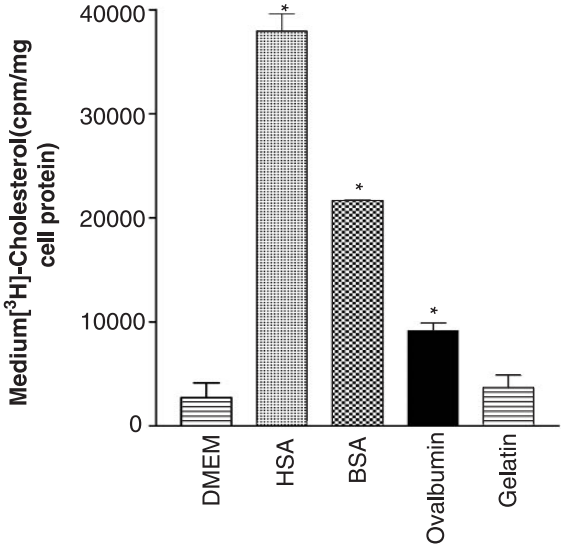


Fig. 2. Effect of HSA, BSA, ovalbumin, and gelatin on cholesterol efflux. Cells were cultured and labeled with [^{3}H]cholesterol for 48 h as described in Fig. 1. Various acceptor proteins (HSA, BSA, ovalbumin, and gelatin) were used at the 10 mg/ml concentration for the efflux study at 90-min incubation time. Results are expressed as radioactivity (cpm) per milligram of cell protein. The values represent the mean ± S.E. from three independent experiments performed in triplicate. *$P < 0.05$ vs. DMEM.

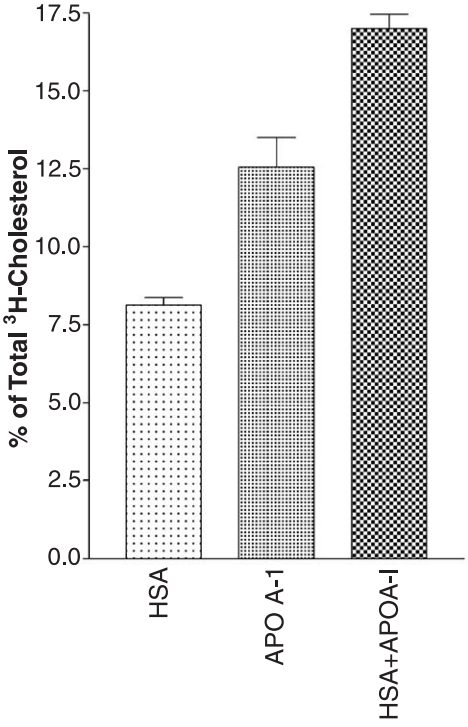


Fig. 3. Effect of apoA-I and HSA on cholesterol efflux. Cells were cultured and labeled as described in Fig. 1, except an equal molar concentration of apoA-I (1 μM), wtrHSA (1 μM), and apoA-I + wtrHSA (1 μM each) were used for the efflux study and compared. Radioactivity released into the media was normalized to milligrams of cell protein and expressed as a percent of total ^{3}H-radioactivity (percent of total efflux=[medium cholesterol/(cell + medium cholesterol)100]). Values represent the mean ± S.E. from three independent experiments performed in triplicate.

lated by various acceptors including HDL, phospholipid, BSA, and is not cAMP dependent. To elucidate whether cAMP is involved in HSA-mediated cholesterol efflux, we added the cell-permeable CPT-cAMP to our endothelial cells and performed the efflux assay. Our results, as shown in Fig. 4, indicated that incubation of endothelial cells with 1 and 2.5 μM HSA in the presence of 0.1 mM CPT-cAMP had no significant stimulatory effect on the efflux rate when compared to cells incubated with HSA without CPT-cAMP. On the other hand, the presence of 0.1 mM CPT-cAMP increased apoA-I-mediated cholesterol efflux by 55% and 44% at 1 and 2.5 μM, respectively, when compared to cells incubated with apoA-I without CPT-cAMP ($P<0.05$ vs. cells without cAMP). These results suggest that HSA-mediated cholesterol efflux is not cAMP dependent and, therefore, may possibly represent a nonspecific diffusion-mediated cholesterol efflux process. To validate our findings, we used the murine RAW264.7 macrophage cell line, which is known to be deficient of ABCA1 protein expression, to study the effects of ABCA1 expression on HSA and apoA-I-mediated cholesterol efflux. cAMP is known to induce apoA-I-mediated cholesterol efflux by stimulating ABCA1 expression in RAW264.7 cells [35]. Our result as shown in Fig. 5 indicated that cAMP did not have any effect on HSA-mediated cholesterol efflux in contrast to apoA-I-mediated cholesterol efflux, confirming our results above with the endothelial cells.

To further characterize the pathway by which HSA mediates cholesterol efflux, the effect of temperature on HSA-mediated cholesterol efflux in [^{3}H]cholesterol-loaded endothelial cells were studied and compared to the apoA-I-

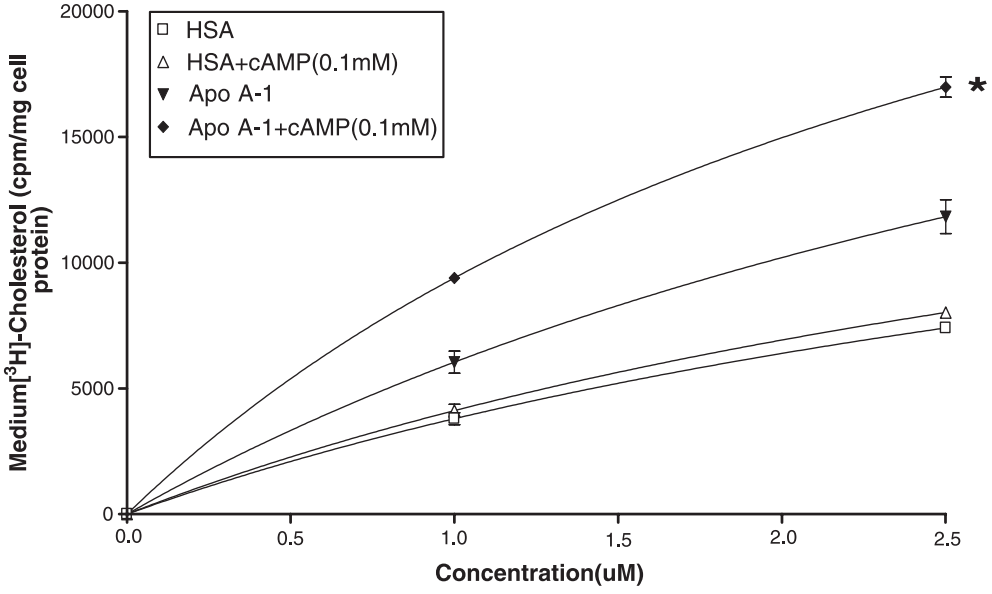


Fig. 4. Effect of cAMP on HSA-mediated cholesterol efflux. EA.hy926 endothelial cells were cultured and labeled as described in Fig. 1. Efflux was performed in the presence of HSA or apoA-I (1 and 2.5 μM) and compared to cells incubated with and without 0.1 mM of cAMP. Results are expressed as cpm/mg cell protein. The values represent the mean ± S.E. from three independent experiments performed in triplicate. *$P<0.05$ vs. cells without cAMP.

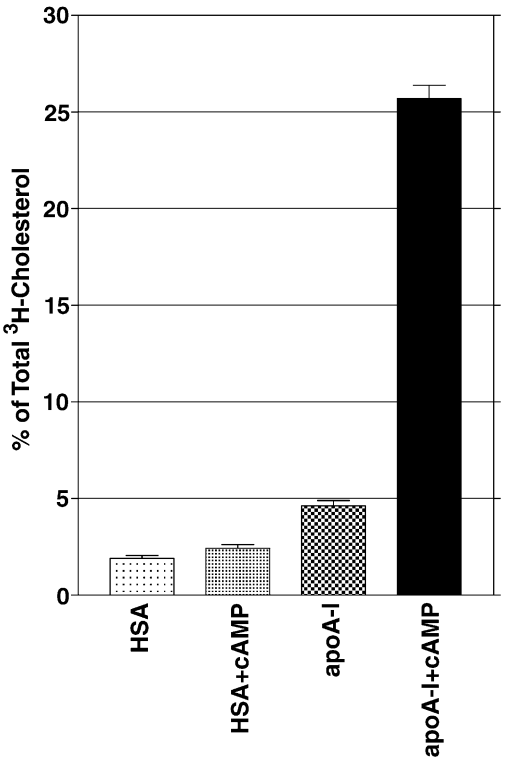


Fig. 5. Effect of cAMP on apoA-I- and HSA-mediated cholesterol efflux from RAW264.7 macrophage cells. Murine RAW264.7 macrophage cells were cultured and labeled as described in Fig. 1. Cells were incubated for 10 h without or with 0.1 mM of cAMP. Cholesterol efflux was measured during subsequent 90-min incubations with 1 μM HSA or apoA-I as cholesterol acceptors. Radioactivity released into the media was normalized to milligrams of cell protein and expressed as a percent of total ^{3}H-radioactivity (percent of total efflux=[medium cholesterol/(cell + medium cholesterol)100]). The values represent the mean ± S.E. from three independent experiments performed in triplicate.

mediated efflux. The apoA-I-mediated process is known to be highly temperature sensitive, since it involves an active transport of intracellular cholesterol to the cell surface via caveolin-1 [31]. On the other hand, nonspecific diffusion-mediated cholesterol efflux has been shown to be less sensitive to temperature changes since it is believed to be passive [31]. As shown in Fig. 6, an Arrhenius plot of ln[percent of total radioactive cholesterol released into the media] versus the reciprocal of temperature showed that HSA-mediated cholesterol efflux was significantly less temperature sensitive compared to the apoA-I-mediated efflux. From the slope of the graph (− Ea/2.3R), activation energy values were calculated for apoA-I-mediated efflux (42.07 kJ/mol) and for the HSA-mediated cholesterol efflux (17.97 kJ/mol), respectively. The fact that an activation energy for apoA-I-mediated process is far greater than that of HSA-mediated process suggests that apoA-I-mediated cholesterol efflux is much more energy dependent than HSA-mediated cholesterol efflux. In summary, the data further support that HSA-mediated cholesterol efflux is independent of the apoA-I-mediated cholesterol efflux process.

3.4. Various HSA mutants display enhanced cholesterol efflux

The diffusion-mediated efflux pathway is largely regulated by the availability of cell surface cholesterol and its affinity to various acceptors of not only lipoproteins, but also plasma proteins. We have shown in this study that HSA can modulate this process. To further characterize the nature of cholesterol–HSA interaction, we examined the effect of various HSA 2A and 3A subdomain mutants on cholesterol efflux and compared to that of the wtrHSA. Endothelial cells were labeled and incubated with 10 mg/ml of each HSA mutants and wtrHSA for 90 min at 37 °C prior to the efflux assay. As shown in Table 1, among the 17 different HSA mutants, the mutants K195M, K199M, F211V, F242V, Y411A, R410A/Y411A, Y411F, W214L/Y411W, and R410Q/Y411W showed increased cholesterol efflux efficiency compared to wtrHSA (117 ± 4%, 109 ± 6%, 120 ± 1%, 112 ± 9%, 116 ± 3%, 108 ± 3%, 135 ± 2%, 134 ± 6%, and 140 ± 4%, respectively vs. wtrHSA). In contrast, HSA mutants W214L, R218A, R218M, R257M, and R410M/Y411F showed decreased cholesterol efflux efficiency compared to wtrHSA (85 ± 3%, 97.4 ± 1%, 90 ± 13%, 89 ± 8%, 84 ± 7%, 93 ± 8%; and 95 ± 7%, respectively). These results indicate that amino acids, Arg at positions 218, 257, and 410, Lys at positions 195 and 199, and Tyr at

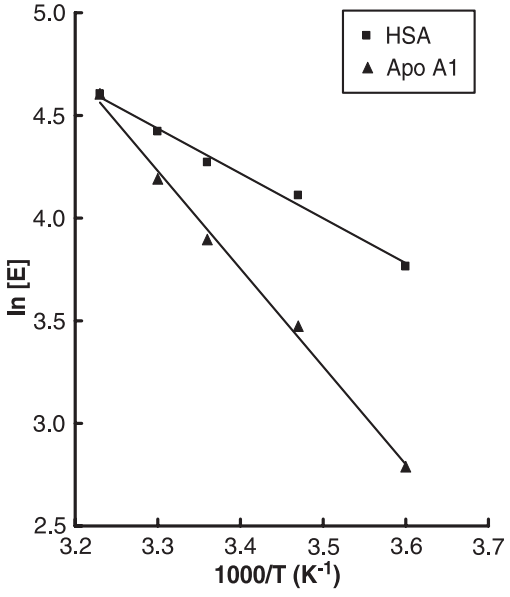
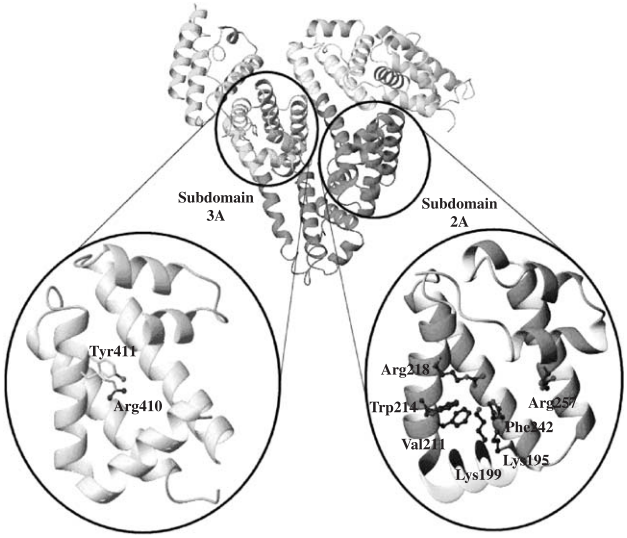


Fig. 6. Arrhenius plot of HSA-mediated and apoA-I-mediated cholesterol efflux. Cells were cultured and labeled as described in Fig. 1. Cells were incubated at temperatures of 4, 15, 25, 30, and 37 °C in DMEM medium containing 1 μM HSA or apoA-I and efflux was determined over a 90-min incubation period. Radioactivity released into the media was normalized to milligrams of cell protein and converted as a percent of total ^{3}H-radioactivity with the 37 °C temperature point set as 100%. For Arrhenius plot, ln *E* was plotted against 1000/*T*, where *E* represents the percent of total ^{3}H-radioactivity and *T* represents absolute temperature. The values represent the mean ± S.E. from three independent experiments performed in triplicate.

Table 1
Subdomain 2A and 3A HSA mutation sites and mutants' effects on cholesterol efflux



	Subdomain 2A mutants							
	K195M	K199M	F211V	W214L	R218A	R218M	F242V	R257M
Cholesterol release into medium, percent of control (wtrHSA)	117.5* ± 5.2	109.0* ± 6.9	119.9* ± 0.4	97.4 ± 0.4	89.7 ± 11.3	88.5* ± 6.9	112.4* ± 9.6	84.2* ± 6.2
	Subdomain 3A mutants							
	Y411A	Y411F	R410A	R410M	W214L/Y411W	R410Q/Y411W	R410A/Y411A	R410M/Y411F
Cholesterol release into medium, percent of control (wtrHSA)	116.0* ± 4.9	135.0* ± 2.9	93.1 ± 11.0	94.4 ± 9.8	133.6* ± 10.3	139.2* ± 8.0	107.5 ± 3.5	83.7* ± 5.3

Cholesterol efflux mediated by HSA mutant proteins were expressed as percent of cholesterol efflux mediated by wtrHSA (set as 100%).
* $P < 0.05$ vs. wtrHSA.

position 411 in subdomains 2A and 3A participate in cholesterol interaction with HSA.

4. Discussion

Many epidemiological studies have shown that high plasma levels of HSA are associated with reduced risk for CHD and mortality from CHD [19–25]. Despite this inverse relationship between HSA levels and CHD, no definitive biochemical mechanisms have been identified to explain this relationship. We have chosen to study the role of HSA on cholesterol efflux as a probable mechanism of action. Previous studies have characterized HSA-mediated cholesterol efflux from cultured human fibroblasts [7–9]. However, the contribution of HSA on cholesterol efflux has not been thoroughly studied, including the mechanism for HSA-mediated cholesterol efflux and the structure–function relationship of HSA on efflux. In our study, we have chosen to investigate the properties of HSA-mediated cholesterol efflux, namely the molecular mechanism for cholesterol efflux and the ligand binding property, using primarily cultured human endothelial cells as the model system. We chose endothelial cells since they are one of the principal cells involved in the cholesterol efflux.

The result of this study clearly provides evidence that HSA plays a role in cholesterol efflux and could be a major contributor in the reverse cholesterol transport. HSA promoted cholesterol efflux in a dose- and time-dependent manner using physiologically relevant concentrations. This effect was shown to be specific as other proteins including gelatin, ovalbumin, and BSA showed either no, little, or moderate effect on cholesterol efflux, respectively. On the other hand, apoA-I-mediated efflux was shown to be superior to that of HSA-mediated efflux. However, considering the high concentration of HSA in plasma (3.5–5 g/dl) and the additive nature of HSA-mediated efflux to apoA-I-mediated efflux, it is speculated that HSA may be a major player in the reverse cholesterol transport from peripheral tissue cells.

Based on previous studies and our findings, we speculated that HSA may harbor at least two binding sites for

cholesterol. These binding sites may not necessarily be specific for cholesterol since HSA is well known to act as a carrier for a large number of hydrophobic ligands including steroid hormones and hydrophobic drugs such as digitalis glycoside, which possess cholesterol-like structure. Using various experimental techniques including spin-labeling, fluorescence, and NMR, these ligands were shown to bind primarily to the 2A or 3A subdomain of HSA [1,13,36]. In particular, one study using chemical modification and fluorescence techniques showed that tryptophan, tyrosine, arginine, and lysine residues of BSA are involved in steroid binding [37]. Our previous studies with HSA mutants reported that substitutions of tryptophan at position 214, phenylalanine at position 211, arginine at positions 218, 222, and 410, lysine at 195 and 199, and tyrosine at position 411 in 2A and 3A subdomains affected HSA interaction significantly with various ligands including thyroxine, warfarin, prostaglandin, digoxin, and bilirubin [11,12,14–16,38–40]. To decipher whether these binding sites of HSA may also be involved in cholesterol binding and thus, in cholesterol efflux, we used a number of polymorphic forms of HSA in our investigation. We chose HSA 2A and 3A mutants based on the presumption that cholesterol would share the same binding sites as with digoxin and steroids to HSA. Interestingly, HSA mutants with substitutions for lysine 195 and 199 (K195M, K199M), phenylalanine 211 and 242 (F211V, F242V), and tyrosine 411 (Y411A, Y411F, W214L/Y411W, R410A/Y411A, and R410Q/Y411W) were more effective in promoting cellular cholesterol efflux when compared to wtrHSA. On the other hand, substitutions of arginine 218 and 257 for methionine (R218M, R257M) or alanine (R218A) were less effective on cholesterol efflux compared to wtrHSA, while substitution of tryptophan 214 for leucine (W214L) exhibited no significant effects on cholesterol efflux. These results suggest that cholesterol interaction with HSA may be similar to the interaction of steroids to HSA since the same amino acid residues were found to have significant effect on steroid and cholesterol binding to the protein. Also, these results are consistent with earlier observations with other HSA ligands such as thyroxine, bilirubin, and warfarin [11,12,14–16,38–40]. Specifically, these results suggest that lysine at position 195 and 199, phenylalanine 211 and 242, and tyrosine at position 411 of wtrHSA resulted in unfavorable effects on cholesterol efflux, so that mutant HSAs with substitutions at these locations had increased cholesterol efflux efficiency. Notably, HSA mutants with substitutions for tyrosine 411 in the 3A subdomain, namely Y411A, Y411F, W214L/Y411W, and R410Q/Y411W, were significantly more effective in promoting cholesterol efflux when compared to wtrHSA. These results are consistent with our earlier observations with digoxin [13]. Interestingly, those HSA mutants in the 3A subdomain, which increased digoxin-binding affinity, also increased cholesterol efflux presumably by enhancing cholesterol-binding affinity. These results suggest that the main interaction between tyrosine 411 with cholesterol might involve the same stacking interactions between the steroid backbone of cholesterol and the aromatic rings of tyrosine, phenylalanine, or tryptophan as seen in previous studies [13].

Another notable observation in our efflux study was with the arginine at position 218 and 410 substitution in the 2A and 3A subdomains. Previous site-directed mutagenesis studies on HSA with various ligands showed that arginine at position 218 in the 2A subdomain, a position homologous to arginine at position 410 in subdomain 3A, had negative interaction with small hydrophobic ligands such as thyroxine, warfarin, and bilirubin [14,16,38]. With a hydrophobic molecule like cholesterol, it is reasonable for us to speculate that replacing arginine at position 218 and 410 with a less polar amino acid residue may enhance the binding of HSA to cholesterol, therefore leading to more efficient cholesterol efflux. In contrast, the substitutions of arginine at position 218 and 410 for methionine (R218M, R218A) or alanine (R410M, R410A) in our study showed the opposite of what we expected based on previous studies with digoxin, warfarin, bilirubin, and thyroxine [14,16,38]. The mutants R218A, R218M, R410A, R410M, and R410M/Y411F showed slightly reduced efflux rates compared with wtrHSA. These results suggest that arginine at positions 218 and 410 are not engaged in cholesterol binding, therefore changing the arginine residues to a more hydrophobic and smaller amino acid did not induce dramatic changes in cholesterol efflux rates.

To examine the mechanism for the cholesterol efflux by HSA, we investigated its effect on diffusion-mediated and apoA-I-mediated cholesterol efflux. In the diffusion-mediated pathway, cholesterol is adsorbed by various acceptors including HDL and BSA [31]. The pathway is said to be nonspecific and passive since it does not involve any specific interaction site on the cell membrane. In the other pathway, cholesterol is removed by lipid-poor HDL via the ABCA1 membrane protein and is cAMP dependent. To differentiate the two possible mechanisms, we measured HSA-mediated cholesterol efflux under different temperature conditions. The apoA-I-mediated process is known to be highly sensitive to temperature change, while diffusion-mediated is less sensitive. Our data supports the diffusion-mediated pathway as HSA-mediated efflux continued at 4 °C temperature, while apoA-I-mediated efflux was virtually abolished. An Arrhenius plot of ln (natural log) efflux rate against 1/temperature suggests that HSA and apoA-I-mediated cholesterol effluxes are single-phase processes and the slope of graphs indicate that apoA-I-mediated cholesterol efflux requires much higher activation energy (42.07 kJ/mol) compared to HSA-mediated efflux (17.97 kJ/mol), supporting HSA-mediated efflux is diffusion-mediated process. To further test this finding, we investigated the effect of cAMP on cholesterol efflux. Since apoA-I-mediated efflux is dependent upon cAMP, cAMP was added to endothelial cells and ABCA1-deficient macrophages. Our results showed cAMP did not stimulate HSA-mediated efflux unlike the

apoA-I-mediated efflux. Taken together, HSA-mediated cholesterol efflux is a distinct process different from the apoA-I-mediated process.

Although it is well accepted that apoA-I-mediated cholesterol efflux involves phospholipids, it is not clear that HSA-mediated efflux also involve phospholipids. Whether HSA–phospholipid interactions may facilitate cholesterol efflux will require further study. However, an earlier study of lysolecithin binding to BSA has shown very weak binding interaction compared to fatty acids [41].

In conclusion, our studies have shown that HSA mediates cholesterol efflux from endothelial cells at physiologically relevant concentrations. This process of efflux may play a significant role in the reverse cholesterol transport from periphery to the liver in maintaining cardiovascular homeostasis. A broader implication of our study supports the many epidemiologic observations that a relatively low level of HSA is a risk factor for cardiovascular disease. The relative contribution and importance of apoA-1-mediated vs. HSA-mediated cholesterol efflux under physiologic and pathologic conditions will require further study. HSA-mediated cholesterol efflux may be quite significant as it has been shown that in ABCA1 knockout mice, cholesterol delivery from periphery to the liver is unchanged compared to controls [42]. At the molecular level, one of the unresolved issues is the nature of cholesterol efflux at the plasma membrane–albumin interphase, which will require further studies.

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